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INHIBITION OF PHOSPHATE UPTAKE IN BARLEY ROOTS BY HYDROXY-BENZOIC ACIDS

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; inhibition of phosphate uptake; hydroxy-benzoic acids; structure–activity relationships.

Abstract—The influence of 15 hydroxy-benzoic acids upon active inorganic phosphate absorption by barley roots was examined. For each compound an inhibition constant (k_i) was determined, i.e. the concentration of compound required to bring about a 50% inhibition of absorption. The k_i values of the benzoic acids were strongly correlated with their octanol–water partition coefficients and their pK_a values. This suggests that the inhibition of normal membrane functions, brought about by benzoic acids, results from a generalized increase in cell membrane permeability. Salicylate derivatives were generally more inhibitory than would be predicted from their partition coefficients; their pronounced toxicity probably arises from structural impediments to their detoxication.

INTRODUCTION

The influence of natural compounds in suppressing the growth and distribution of species (the phenomenon of allelopathy) is considered to be extremely important in the evolution of plant communities [1–3]. Prominent amongst the classes of chemicals involved in allelopathic interactions are phenolics because of their variety and ubiquity in the environment and their pronounced biological activity [1]. Hydroxy-benzoic and -cinnamic acids, in particular, have been identified from the tissues of all higher plants [3]. Through various mechanisms they are introduced into soils in sufficient concentrations to be biologically active [2].

It has been shown that benzoic acids are capable of inhibiting active potassium [4] and phosphate absorption [5] as well as depolarizing cell membrane electrical potentials [6]. These effects are instantaneous, and usually readily reversible following short exposures to the compounds in question. Longer exposures, at physiologically meaningful concentrations, have been shown to permanently impair membrane function [7]. This paper examines the inhibition of phosphate absorption by 15 benzoic acids, and considers their relative potency for disrupting membrane function in terms of their structure and metabolism in plants and animals.

RESULTS AND DISCUSSION

Table 1 shows the k_i values for the 15 benzoic acids employed in this study, together with the 95% confidence limits of the estimates, the logs of their partition coefficients between octanol and water (Log P) and their pK_a values. There was considerable variation in the inhibitory capacity of these compounds ranging from *o*-hydroxybenzoic acid (salicylate), k_i $1{\cdot}85 \times 10^{-4}$, to 3,4,5-trihydroxybenzoic acid (gallic acid), k_i $1{\cdot}66 \times 10^{-3}$ M. Figure 1 shows a plot of k_i values against log P values. The numbers associated with each point correspond to the number employed to identify the compound in Table 1. Since the concentrations of inhibitors required to reduce absorption by 50% decrease with increasing toxicity, the k_i values and log P values are negatively correlated. This relation is expressed in the equation: $k_i = 21{\cdot}004 - 8{\cdot}351 \log P$, where k_i = inhibition constant and log P = log of the partition

Table 1. Inhibition constants (k_i) of the benzoic acids together with the 95% confidence limits of these estimates, the logs of the partition coefficients and the pK_a values.

Compound tested	$k_i \times (10^{-4}$ M) with 95% confidence limits		Log P	pK_a
1 Benzoic acid	4·97	3·69–6·58	1·87	4·19
Benzoic acid derivative				
2 *o*-OH	1·85	0·85–2·76	2·21	2·97
3 *p*-OH	7·47	7·31–7·86	1·58	4·48
4 2,3-diOH	2·61	2·20–3·19	1·83	2·97
5 2,4-diOH	8·75	.7·3–9·30	1·60	4·70
6 2,5-diOH	3·90	3·67–4·70	1·72	2·97
7 2,6-diOH	5·00	3·16–6·79	2·20	2·70
8 3,4-diOH	12·4	12·1–14·0	1·20	4·48
9 3,5diOH	8·89	7·62–10·26	1·40	4·04
10 3,4,5-triOH	16·6	13·8–19·5	0·83	4·41
11 *m*OMe	4·19	3·64–4·77	1·95	4·09
12 3,4,5-tri-OMe_3	5·10	4·49–5·66	2·23	4·228
13 5-OMe,2-OH	2·38	1·47–3·25	2·41	2·86
14 3-OMe,4-OH	2·87	2·22–3·52	1·72	4·432
15 3,5-diOMe,4-OH	6·25	5·76–6·73	1·85	4·317

coefficient of the benzoic acid between octanol and H_2O.

Statistical analysis of this data revealed a correlation coefficient of −0·866. Analysis of variance established that the correlation was highly significant at the 0·01 level of confidence. The correlation coefficient value of −0·866 demonstrates that log P values account for 75% of the variability in the data. A regression of k_i values against pK_a values showed a significant correlation (0·58) at the 0·05 level of confidence. Nevertheless a multiple linear regression analysis showed that the addition of pK_a values did not significantly improve the prediction power of the regression equation based solely upon log P values. Thus k_i values are probably related to pK_a values only in so far as log P values are influenced by the degree of dissociation of the benzoic acids. This information strongly supports the hypothesis, derived earlier from kinetic studies [5], that inhibition of ion absorption and depolarization of cell membrane electrical potentials by benzoic acids is determined by their solubility in the lipid components of the cell membrane. Presumably, the presence of these compounds in the membrane causes rapid and reversible changes in permeability to both cations and anions.

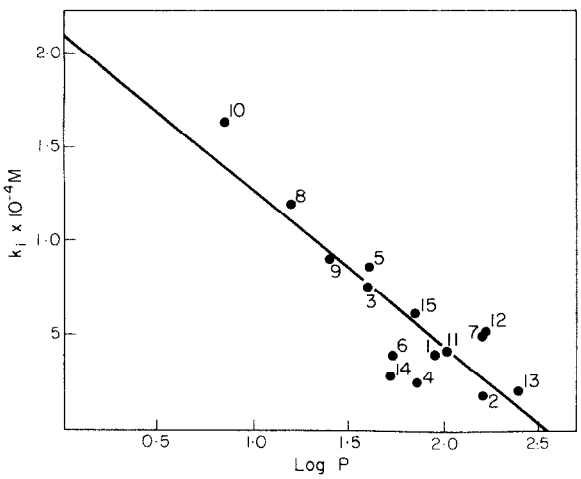

Fig. 1. The k_i (inhibition constants) are shown as a function of log P values. The numbers adjacent to the points refer to the compounds listed in Table 1.

Studies with molluscan neurones have shown that benzoic acid derivatives cause hyperpolarization of membrane potentials [8]. However the concentrations required to produce significant changes in potential were of the order of 1×10^{-2} M (at least 100× the concentration required to completely depolarize root cell membrane potentials). The results of these animal studies were interpreted to suggest that the impairment of normal membrane function by benzoic acids could be accounted for solely on the basis of permeability changes. However in the plant studies under discussion the fact that a sizeable proportion of the variability in data in not accounted for by log P values (25% in fact), points to an additional, possibly metabolic, component of inhibition.

Phenolic acids such as salicylate have been

Table 2. Observed k_i values as well as predicted values (from the regression equation) and the residual values (observed–predicted) of the benzoic acids

Compound tested	k_i value × 10^{-4} M Actual	Predicted	Residual
Benzoic acid	4·970	5·388	−0·418
Benzoic acid derivative			
o-OH	1·850	2·549	−0·699
p-OH	7·470	7·810	−0·340
2,3-diOH	2·610	5·722	−3·112
2,4-diOH	8·750	7·643	+1·107
2,5-diOH	3·900	6·641	−2·741
2,6-diOH	5·000	2·632	+2·368
3,4-diOH	12·100	10·983	+1·117
3,5-diOH	8·890	9·313	−0·423
3,4,5-triOH	16·600	14·073	+2·527
m-OMe	4·190	4·720	−0·530
3,4,5-triOMe	5·100	2·382	+2·718
5-OMe,2-OH	2·380	0·879	+1·501
3-OMe,4-OH	2·870	6·641	−3·771
3,5-diOMe,4-OH	6·250	5·555	+0·695

shown to be effective uncouplers of oxidative phosphorylation [9]. According to the Mitchell Chemiosmotic hypothesis of phosphorylation [10] uncoupling agents are considered to act by causing permeability changes which result in the discharge of electrochemical potential gradients across the mitochondrial membrane. Thus both a direct effect upon cell membrane permeability and a short circuiting of ATP for ion uptake could be based upon the same mechanism of action.

Examination of the column headed Residual (Table 2) shows that certain of the *o*-hydroxylated derivatives are considerably more potent inhibitors of phosphate absorption than would be predicted from the regression equation. Consideration of the metabolism of these compounds by plants and animals, as well as their molecular configurations provide an explanation for their additional activity.

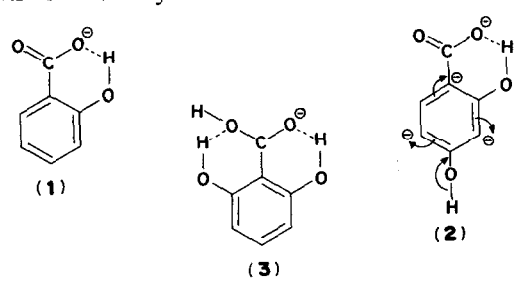

Fig. 2. Chelation effects produced by interaction between the carboxylate ion and the *o*-hydroxyl group of salicylate (**1**) 2,4-dihydroxybenzoic acid, (**2**) and 2,6-dihydroxybenzoic acid (**3**).

Phenolic compounds administered to higher plants are readily detoxified by the formation of conjugates with glucose [11]. Animals respond in like fashion with the formation of glucuronic acid or sulphate derivatives [12]. All these derivatives are less lipid-soluble than the parent phenolic, and hence less likely to be injurious to membranous structures. While most benzoic acids are normally esterified at the carboxyl group, with glucose, salicylate derivatives are differently metabolized by plants [13]. In *Gaultheria procumbens*, which contains large quantities of methyl salicylate, exposure to salicylate resulted in the death of leaf tissue, although 3,4-dihydroxybenzoate was esterified and the tissue remained healthy. The major derivative recovered from tissue to which salicylate had been administered was a 2,6-dihydroxybenzoate conjugate. Thus hydroxylation at the 6 position preceded conjugation with glucose. Examination of Table 1 shows that this substitution markedly reduces the toxicity of salicylic acid (k_i values of $1{\cdot}85 \times 10^{-4}$ M and $5{\cdot}00 \times 10^{-4}$ M for salicylic acid and 2,6-dihydroxybenzoic acid respectively). Furthermore, as a group, *o*-hydroxylated compounds were converted to glucosides rather than glucose esters. When salicylate is fed to animals it is excreted, largely without sulphate formation and limited glucuronide synthesis [12]. Similarly, esterification of *o*-hydroxylated derivatives of benzoic acid is difficult compared to benzoic acid itself [14]. Thus esterification, the usual means of detoxication, is somehow impeded in the case of salcylate derivatives.

The stronger acidity and greater lipid solubility of salicylate, compared to benzoic acid, has been rationalized on the basis of the formation of a stable six-membered chelate by hydrogen binding between the phenolic hydroxyl proton and the carboxylate carbonyl (Fig. 2) [14]. Subsequent hydroxylation of salicylate may reinforce or undermine the stability of this hydrogen bond effect according to location. Thus at positions 4 or 6, resonance contribution destabilizes the structure with resulting decreased lipid solubility and acidity. Residual values (actual k_i–predicted k_i: Table 2) are positive for these compounds. They are less potent than predicted on the basis of log *P*. At positions 3 or 5, resonance contribution cannot destabilize the chelation effect. These compounds have high log *P* values, low pK_a values and nega-

tive residual values. This hydrogen bond effect almost certainly results in decreased facility for esterification by stabilizing the carboxylate ion and providing steric impediments to enzymic transglucosylation. Factors which destabilize this structure reverse these effects with reduced toxicity for the compound concerned.

The present report establishes that the major factor in the inhibition of phosphate uptake by the benzoic acids is their lipid solubility. This information, taken with previous kinetic studies support the hypothesis of direct permeability effects upon the cell membrane. The statistical analysis reveals that a significant contribution to the biological activity of these compounds may be based upon a metabolic component which, in the case of the *o*-hydroxylated compounds, is almost certainly due to structural impediments to detoxication.

EXPERIMENTAL

Plant material. Barley roots were prepared for absorption experiments according to the methods described previously [5]. When 4 days old, the roots were excised and weighed into 2 g portions.

Determination of phosphate absorption. Root samples were immersed in 150 ml beakers containing 50 ml of incubation soln containing 5×10^{-5} M P_i, 5×10^{-4} M $CaCl_2$ and 0·05 μc of ^{32}P-labelled phosphate. All solns were buffered at pH 7 and maintained at 23° during the uptake period. Various hydroxy-benzoic acids were added to the incubation soln dissolved in 1 ml of 95% EtOH. During the uptake periods the solns were aerated continuously. Aliquots of the incubation media were removed at various intervals of time and the radioactivity of these samples determined in a Packard scintillation spectrometer. By this means the rates of absorption of radioactivity were determined. Inhibition values quoted in this study are based upon 3 hr uptake periods.

Calculation of k_i *values.* For each compound, the % inhibition of phosphate uptake was determined at several concentrations of inhibitor. The regression line for inhibition on concentration was determined and from this line the concentration corresponding to a 50% inhibition of absorption (k_i) obtained.

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